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Insulin-like Growth Factor 1 Receptor Signaling Is Required for Optimal ATR-CHK1 Kinase Signaling in Ultraviolet B (UVB)-irradiated Human Keratinocytes*

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UVB wavelengths of light induce the formation of photoproducts in DNA that are potentially mutagenic if not properly removed by the nucleotide excision repair machinery. As an additional mechanism to minimize the risk of mutagenesis, UVB-irradiated cells also activate a checkpoint signaling cascade mediated by the ATM and Rad3-related (ATR) and checkpoint kinase 1 (CHK1) kinases to transiently suppress DNA synthesis and cell cycle progression. Given that keratinocytes in geriatric skin display reduced activation of the insulin-like growth factor 1 receptor (IGF-1R) and alterations in DNA repair rate, apoptosis, and senescence following UVB exposure, here we used cultured human keratinocytes *in vitro* and skin explants *ex vivo* to examine how IGF-1R activation status affects ATR-CHK1 kinase signaling and the inhibition of DNA replication following UVB irradiation. We find that disruption of IGF-1R signaling with small-molecule inhibitors or IGF-1 withdrawal partially abrogates both the phosphorylation and activation of CHK1 by ATR and the accompanying inhibition of chromosomal DNA synthesis in UVB-irradiated keratinocytes. A critical protein factor that mediates both ATR-CHK1 signaling and nucleotide excision repair is replication protein A, and we find that its accumulation on UVB-damaged chromatin is partially attenuated in cells with an inactive IGF-1R. These results indicate that mutagenesis and skin carcinogenesis in IGF-1-deficient geriatric skin may be caused by defects in multiple cellular responses to UVB-induced DNA damage, including through a failure to properly suppress DNA synthesis on UVB-damaged DNA templates.

The major risk factor for the development of non-melanoma skin cancer (NMSC)[2] is the exposure to UV wavelengths of sunlight, which generate photoproducts in genomic DNA that give rise to mutations when DNA polymerases incorrectly copy the damaged bases. Nucleotide excision repair is the sole mechanism for removing these photoproducts from genomic DNA in human cells (1, 2), and mutations in excision repair genes give rise to the skin cancer-prone disease xeroderma pigmentosum and to the premature aging disease Cockayne syndrome (3, 4).

In addition to DNA repair, UV-irradiated cells possess DNA damage checkpoint signaling cascades that inhibit DNA synthesis on damaged templates to limit the likelihood of mutagenesis. This suppression of DNA synthesis occurs through a number of mechanisms, including by transiently preventing cells from entering the S phase of the cell cycle, inhibiting the initiation of DNA replication at new origins, and slowing the rate of ongoing replication fork progression (5–7). In addition to directly preventing the miscopying of damaged DNA bases, these checkpoint responses also allow additional time for DNA repair to take place prior to the resumption of DNA synthesis.

Critical components of this UV-induced DNA damage signaling response are the protein kinases ATM and Rad3-related (ATR) and checkpoint kinase 1 (CHK1) (8–11). CHK1 is directly phosphorylated and activated by ATR (12), and, together, these proteins phosphorylate numerous additional proteins associated with DNA replication and cell cycle progression (13, 14). In UV-irradiated cells, the signal that triggers the activation the ATR-CHK1 pathway is thought to be regions of single-stranded DNA (ssDNA) produced by replicative helicase-polymerase uncoupling (15) and by gaps generated during nucleotide excision repair (16–18). This ssDNA is thought to then become bound by replication protein A (RPA), which coordinates many aspects of ATR-CHK1 signaling (19–21), including the recruitment of the ATR kinase (22), its activator module comprised of the 9-1-1 clamp and TopBP1 (23–26), and the adaptor proteins Timeless, Tipin, and Claspin, which specifically facilitate the phosphorylation of CHK1 (27). The importance of the ATR-CHK1 pathway in DNA damage responses and carcinogenesis is highlighted by mouse genetic studies in which loss of one allele of either ATR or CHK1 has

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[2] The abbreviations used are: NMSC, non-melanoma skin cancer; ATR, ATM and Rad3-related; ssDNA, single-stranded DNA; RPA, replication protein A; UVR, UV radiation; IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptor; PPP, picropodophyllin.

been shown to promote tumorigenesis (28–31), including in the skin (32).

There are an abundance of genetic and environmental factors that may influence DNA repair, mutagenesis, and carcinogenesis associated with exposure to UV radiation (UVR). However, the observation that more than 80% of NMSCs are found in patients over the age of 60 (33, 34) has indicated that age and the physiology of aged skin may contribute to the propensity for skin carcinogenesis.

Consistent with this hypothesis, the age-dependent accumulation of senescent fibroblasts in the dermis of the skin may lead to a tumor-promoting environment that influences how keratinocytes in the epidermis respond to environmental carcinogens such as UVR. Indeed, previous studies have shown that the production of insulin-like growth factor 1 (IGF-1), which is primarily supplied to epidermal keratinocytes by dermal fibroblasts (35, 36), is decreased in both senescent fibroblasts *in vitro* and geriatric skin *in vivo* (37). The observation that epidermal keratinocytes in geriatric skin display reduced activation of the IGF-1 receptor (IGF-1R) further supports the notion that the IGF-1/IGF-1R axis becomes disrupted as people age (37, 38).

Interestingly, previous *in vitro* and *in vivo* studies with cultured keratinocytes and human skin have shown that the IGF-1/IGF-1R axis influences both the UVB photoproduct removal rate (39, 40) and various cellular outcomes following UVB exposure, including apoptosis and senescence (37, 40–42). Because the ATR-CHK1 pathway is an additional and important regulator of the cellular response to UVR-associated DNA damage, we have now examined how the status of the IGF-1R influences the activity of the ATR-CHK1 signaling pathway.

Using cultured human keratinocytes *in vitro* and skin explants *ex vivo*, we find that maximal activation of the ATR-CHK1 kinase network and suppression of DNA synthesis after UVB irradiation requires an intact IGF-1/IGF-1R system. Importantly, we observed that RPA, which plays critical roles in both ATR-CHK1 signaling (19, 20) and in UV photoproduct removal by nucleotide excision repair (43, 44), fails to properly accumulate on UVB-damaged chromatin in cells exposed to an IGF-1R inhibitor. Together, these results suggest that defects in both nucleotide excision repair and ATR-CHK1 checkpoint signaling may contribute to aging-associated mutagenesis and carcinogenesis in human skin.

Results

IGF-1R Inhibition Partially Abrogates ATR-CHK1 Signaling and the Suppression of DNA Synthesis in UVB-irradiated, Telomerase-immortalized Human Keratinocytes in Vitro—Given the well described roles of the ATR and CHK1 kinases in suppressing DNA synthesis in UV-irradiated cells (8, 9, 11) and the recognition that the activation status of the IGF-1R affects the fate of UVB-irradiated keratinocytes *in vitro* and *in vivo* (37, 39, 41, 42), we treated N-TERT keratinocytes with an IGF-1R inhibitor for 1 h prior to exposure to UVB radiation and then monitored the kinetics of CHK1 phosphorylation. As shown in Fig. 1*A* (*lanes 1–6*), exposure of cells to 500 J/m^2 of UVB induced a robust and transient phosphorylation of CHK1 on the ATR target residue Ser-345 that peaked 0.5 h following irradiation. Although UVB-irradiated cells that were pretreated with the IGF-1R inhibitor AG538 were still capable of inducing CHK1 phosphorylation with similar general kinetics as control cells, the extent of phosphorylation was reduced by ~3- to 4-fold (Fig. 1*A*, *lanes 7–12*). Importantly, this defect in ATR kinase signaling was not specific to its target CHK1, however, as we also observed that phosphorylation of the ATR substrate KAP-1 was also partially abrogated by inhibition of the IGF-1R.

To examine these findings further with UVB doses that do not induce significant amounts of apoptosis in these cells (37), we repeated the experiment using a range of lower UVB exposures. As shown in Fig. 1*B*, regardless of the UV light dose, the level of CHK1 and KAP-1 phosphorylation was reduced by up to 3-fold on average when cells were treated with the IGF-1R inhibitor AG538. We conclude that the UVB-induced activation of ATR and CHK1 is disrupted when the IGF-1R is inhibited in human keratinocytes.

Through the targeting of proteins that promote S phase entry, replication origin activation, and replication fork progression and stability, the ATR-CHK1 pathway actively and transiently suppresses DNA synthesis in cells exposed to UVR and other DNA-damaging agents (8, 9, 11). Thus, to determine whether the abrogation of ATR-CHK1 signaling observed in cells treated with an IGF-1R inhibitor was associated with an altered rate of DNA synthesis, we measured DNA replication using a BrdU immuno-dot blot assay (47). As shown in Fig. 1*C*, DNA synthesis was inhibited in a UVB dose-dependent manner in both DMSO- and AG538-treated cells. However, in the presence of an IGF-1R inhibitor, DNA synthesis failed to be suppressed to the extent observed in control cells.

At low fluences of UV irradiation, the inhibition of DNA synthesis is only transient, and cells are able to recover and resume DNA replication. Consistent with this classical response, maximal inhibition of DNA replication occurred 1–2 h after UVB exposure, and the DNA synthesis rate ultimately returned to near normal levels by 6.5 h post-UVB irradiation (Fig. 1*D*). In cells treated with the IGF-1R inhibitor, however, DNA synthesis was only partially suppressed. Thus, consistent with the analysis of ATR-CHK1 signaling, DNA synthesis failed to be properly stopped following UVB exposure in cells with an inactive IGF-1R.

IGF-1R Status Impacts ATR-CHK1 Signaling and DNA Replication Inhibition in Primary Keratinocytes—To confirm the results presented in Fig. 1 with non-transformed cells, we obtained primary keratinocytes from human foreskins and then repeated our measurements of ATR-CHK1 signaling and DNA synthesis using two different inhibitors of the IGF-1R. As shown in Fig. 2*A*, treatment of primary keratinocytes with the IGF-1R competitive inhibitor AG538 or non-competitive inhibitor PPP reduced UVB-induced CHK1 phosphorylation by ~50% in comparison with DMSO-treated cells. Similarly, when we examined the UVB-induced inhibition of DNA synthesis with a BrdU immuno-dot blot assay, we observed that keratinocytes treated with the IGF-1R inhibitors failed to inhibit DNA synthesis to the extent found in control, DMSO-treated cells. Together, these results suggest that IGF-1R inhibition interferes with ATR-CHK1 kinase signaling and DNA replication suppression in both primary and telomerase-immortalized UVB-irradiated human keratinocytes.

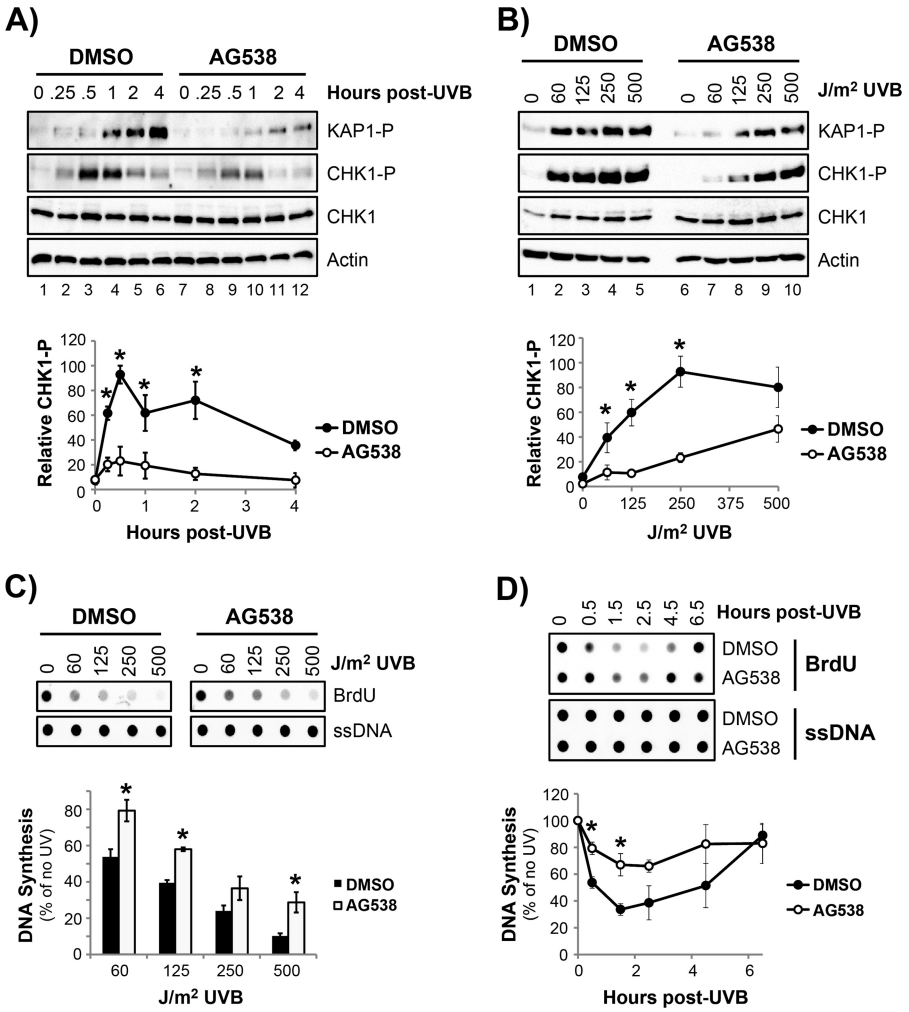

FIGURE 1. **Inhibition of the IGF-1/IGF-1R pathway in N-TERT keratinocytes partially abrogates ATR-CHK1 signaling and the suppression of DNA synthesis following UVB irradiation *in vitro*.** *A*, TERT-immortalized human keratinocytes were treated for 1 h with DMSO or the IGF-1 receptor inhibitor AG538 (10 μM) prior to exposure to 500 J/m^2 of UVB radiation. Cells were harvested at the indicated time points, and cell lysates were examined by immunoblotting. The quantitation shows the results from three independent experiments in which phospho-CHK1 (CHK1-P, Ser-345) signals were normalized to total CHK1. The highest CHK1-P/CHK1 ratio for each blot was set to an arbitrary value of 100. All other signals were normalized to this value, and the mean ± S.E. are shown. The *asterisks* indicate that CHK1-P phosphorylation in the DMSO- and AG538-treated cells are significantly different (*, $p < 0.05$). *B*, cells were treated as in *A*, except that cells were exposed to different fluences of UVB light and then harvested 1 h post-irradiation. The quantitation shows results from four independent experiments. The *asterisks* indicate a significant difference in CHK-1 phosphorylation at the indicated time points (*, $p < 0.05$). *C*, cells were treated as in *B*, except that BrdU (10 μM) was added to the culture medium immediately after irradiation with the indicated doses of UVB light. Cells were harvested 0.5 h later. Genomic DNA was purified, dot-blotted onto a nitrocellulose membrane, and probed with an anti-BrdU antibody. Blots were reprobed with an anti-ssDNA antibody to ensure equal loading. The quantitation shows results (mean ± S.E.) for each UVB dose from two to five independent experiments in which BrdU signals for UVB-irradiated cells were normalized to a non-irradiated control. The asterisks indicate that DNA synthesis in the AG538-treated cells is significantly different from in the DMSO-treated cells ($p < 0.05$). *D*, Cells exposed to 60 J/m^2 of UVB radiation were analyzed as in *C*, except that BrdU was added 0.5 h prior to cell harvesting at the indicated time points. The quantitation shows the results from three to five independent experiments for each time point. The *asterisks* indicate a significant difference in DNA synthesis at the indicated time points (*, $p < 0.05$).

IGF-1 Is Required for Optimal ATR-CHK1 Signaling—We next examined how the presence or absence of IGF-1 in the culture medium affected the response of keratinocytes to UVB. We therefore starved N-TERTs of IGF-1 for 18–24 h and then supplemented the culture medium with recombinant IGF-1 or PBS vehicle for 1 h prior to exposure to UVB. As shown in Fig. 3*A*, a more robust phosphorylation of CHK1 was observed in cells that had been exposed to IGF-1 than in cells starved of IGF-1.

We next examined how the presence or absence of IGF-1 affected the inhibition of DNA synthesis following UVB irradiation. We observed that the inhibition of DNA replication in UVB-irradiated keratinocytes was dependent on the concentration of IGF-1 in the culture medium (Fig. 3*B*). Experiments monitoring the kinetics of DNA synthesis following UVB irradiation showed that the inhibition of DNA synthesis at early time points following UVB exposure was significantly greater in cells exposed to IGF-1 than cells starved of IGF-1 (Fig. 3*C*).

Thus, these results demonstrate that both IGF-1 withdrawal and IGF-1R inhibition have qualitatively similar effects on ATR-CHK1 signaling and DNA synthesis following UVB exposure. The quantitative differences observed between these two experimental approaches likely arise from the effects of prolonged withdrawal of an essential keratinocyte growth factor on cell cycle distribution, which, among other effects, may impact the relative the contribution of helicase-polymerase uncou-

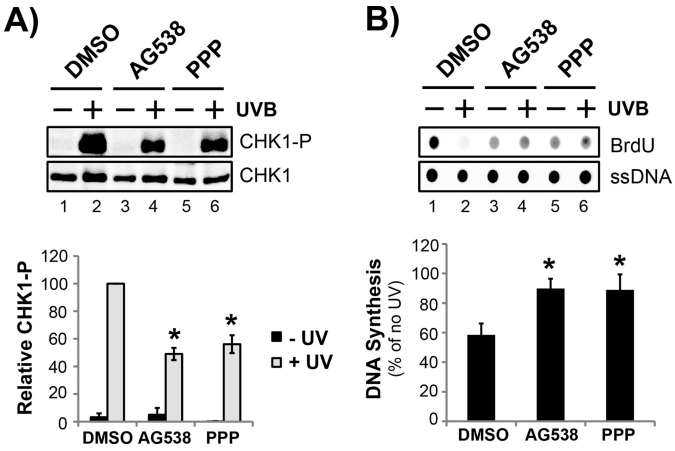

FIGURE 2. **Optimal activation of ATR-CHK1 signaling and replication inhibition in UVB-irradiated primary normal human keratinocytes requires the IGF-1/IGF-1R system.** *A*, primary human foreskin keratinocytes were treated for 1 h with DMSO or the IGF-1 receptor inhibitors AG538 or PPP before exposure to 60 J/m^2 of UVB radiation. Cells were harvested 0.5 h later and analyzed by immunoblotting. The graph shows the results (mean ± S.E.) from three independent experiments with three different foreskin keratinocyte samples. *B*, primary keratinocytes treated with DMSO, AG538, or PPP were exposed to 60 J/m^2 and then incubated with BrdU for 0.5 h. Genomic DNA was analyzed by immuno-dot-blotting as in Fig. 1. Results show the mean ± S.E. from three independent experiments. The *asterisks* indicate that CHK1 phosphorylation or DNA synthesis in the AG538-treated cells is significantly different from in the DMSO-treated cells (*, $p < 0.05$).

pling and nucleotide excision repair gaps to ATR-CHK1 activation. Furthermore, we noted that withdrawal of IGF-1 for 18–24 h led to a modestly reduced rate of basal DNA synthesis in non-irradiated cells (data not shown).

IGF-1R Inhibition Abrogates ATR-CHK1 Signaling in Human Skin Explants ex Vivo—To extend our *in vitro* findings with keratinocytes to the more physiological environment of whole skin, we used deidentified skin tissue derived from human skin contour surgeries (abdominoplasties). As shown in Fig. 4*A*, exposure of human skin to UVB light led to a dose-dependent increase in the phosphorylation of CHK1 on both an ATR target site (Ser-345) and an autophosphorylation site (Ser-296) within the epidermis of the skin. Examination of the kinetics of CHK1 phosphorylation showed that CHK1 phosphorylation peaked 0.5–1 h after UV irradiation (Fig. 4*B*), similar to that observed in keratinocytes *in vitro*. Importantly, these results provide the first experimental evidence that the ATR-CHK1 pathway can be activated in the epidermis of human skin exposed to UVB.

We next examined how inhibition of the IGF-1R affected this response to UVB radiation. We therefore topically applied either DMSO or the IGF-1R inhibitor AG538 to skin samples 0.5 h prior to exposure to UVB light and then harvested the epidermis 0.5–1 h after irradiation. As shown in Fig. 4*C*, UVB irradiation-induced CHK1 and KAP-1 phosphorylation was partially abrogated in the presence of the IGF-1R inhibitor. Quantitation of several independent experiments with different skin samples demonstrated that CHK1 phosphorylation was reduced by more than 60% at high doses of UVB light. These results show that, similar to human keratinocytes *in vitro*, the status of the IGF-1R affects the activation of the ATR-CHK1 pathway in keratinocytes within the context of the human epidermis *ex vivo*.

IGF-1R Inhibition Interferes with Proper RPA Loading onto UVB-damaged Chromatin—To characterize the mechanism by which the ATR-CHK1 pathway is disrupted by the inhibition of the IGF-1R, we next considered how the status of the IGF-1R affects the accumulation of RPA on UVB-damaged chromatin. RPA plays multiple roles in ATR-CHK1 signaling by directly interacting with several components of the signaling reaction, including the ATRIP subunit of the ATR kinase holoenzyme (19, 22), the ATR kinase-activating 9-1-1 clamp and TopBP1 module (23, 24), and Tipin, which, through binding to the adaptor protein Claspin, is thought to facilitate the recruitment of CHK1 to ATR (27).

As expected, we observed that RPA rapidly accumulated on the chromatin fraction of UVB-irradiated N-TERT cells and peaked ~1 h after UVB exposure (Fig. 5*A*, *lanes 1–5*). However, in cells treated with the IGF-1R inhibitor AG538, we noted two relevant observations. First, the level of chromatin-bound RPA was elevated nearly 2-fold even prior to UVB exposure. Second, the chromatin level of RPA then failed to undergo a significant increase following UVB exposure. These results indicate that an intact IGF-1R signaling network is required for RPA to properly function in promoting the ATR-CHK1 pathway following UVB irradiation.

Discussion

The efficient removal of UVB photoproducts from genomic DNA by the nucleotide excision repair machinery is critical to the fate of UVB-irradiated cells; incomplete repair is associated with a number of potential outcomes, including mutagenesis, senescence, and apoptosis. Although the nucleotide excision repair system is the sole mechanism for removing the photoproducts from DNA (1, 2), additional DNA damage response signaling proteins, including the ATR and CHK1 protein kinases, also influence cellular responses to UVR (5–9).

The classical functions of the ATR and CHK1 kinases include the induction of a transient suppression of DNA synthesis and the activation of cell cycle checkpoints (8–10). However, ATR has also been reported to facilitate UV photoproduct removal through direct (48–51) and indirect means (52, 53). ATR may also induce cellular senescence (54, 55) and promote or prevent apoptosis (47), depending on the specific stimulus. Given that the activation status of the IGF-1R also influences these outcomes in UVB-irradiated human keratinocytes (37–39, 41, 42) and is known to be deficient in geriatric skin (37), determining how IGF-1/IGF-1R signaling influences ATR-CHK1 pathway function in UVB-irradiated keratinocytes may uncover novel insights into the mechanism of aging-associated skin carcinogenesis.

Using IGF-1 ligand withdrawal and small-molecule inhibitors of the IGF-1R, we showed here that inactivation of the IGF-1R is associated with a partial disruption of ATR-CHK1 signaling and DNA synthesis inhibition in UVB-irradiated keratinocytes. The single-stranded DNA-binding protein RPA interacts with several components of the ATR-CHK1 signal transduction network, and thus its failure to accumulate on chromatin following UVB exposure provides a logical mechanistic explanation for the observed defects in DNA damage signaling. Moreover, RPA is also one of the six essential compo-

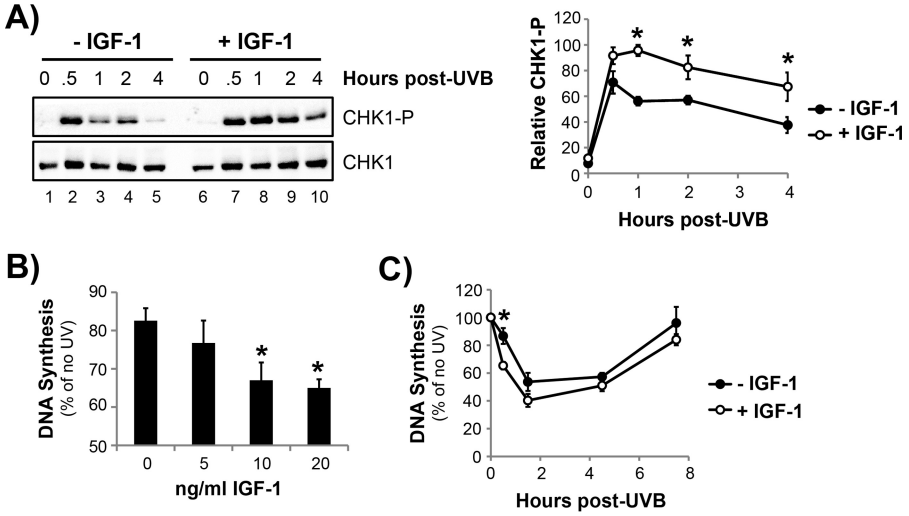

FIGURE 3. **IGF-1 is required for UVB-induced ATR-CHK1 signaling and inhibition of DNA synthesis in human keratinocytes.** *A*, N-TERT keratinocytes starved of IGF-1 for 18–24 h were treated with PBS or 20 ng/ml recombinant IGF-1 for 1 h before exposure to 60 J/m^2 of UVB. Cell lysates were analyzed by immunoblotting. The quantification shows results (mean ± S.E.) from three independent experiments. *B*, N-TERTs starved of IGF-1 as in *A* were exposed to the indicated concentrations of recombinant IGF-1 for 1 h. Cells were then exposed to 60 J/m^2 of UVB radiation, incubated with BrdU, and harvested 0.5 h later. Genomic DNA was isolated and analyzed by immuno-dot-blotting. The graph shows the mean (± S.E.) amount of DNA synthesis relative to non-irradiated cells from four to ten independent experiments. *C*, cells deprived of IGF-1 or exposed for 1 h to 20 ng/ml of recombinant IGF-1 were exposed to UVB light and then incubated for 0.5 h with BrdU immediately prior to harvesting at the indicated time points. Genomic DNA was analyzed by immuno-dot-blotting. The results indicate the relative level of DNA synthesis at each time point from three separate experiments. The *asterisks* indicate that CHK1 phosphorylation or DNA synthesis in the AG538-treated cells is significantly different from in the DMSO-treated cells (*, $p < 0.05$).

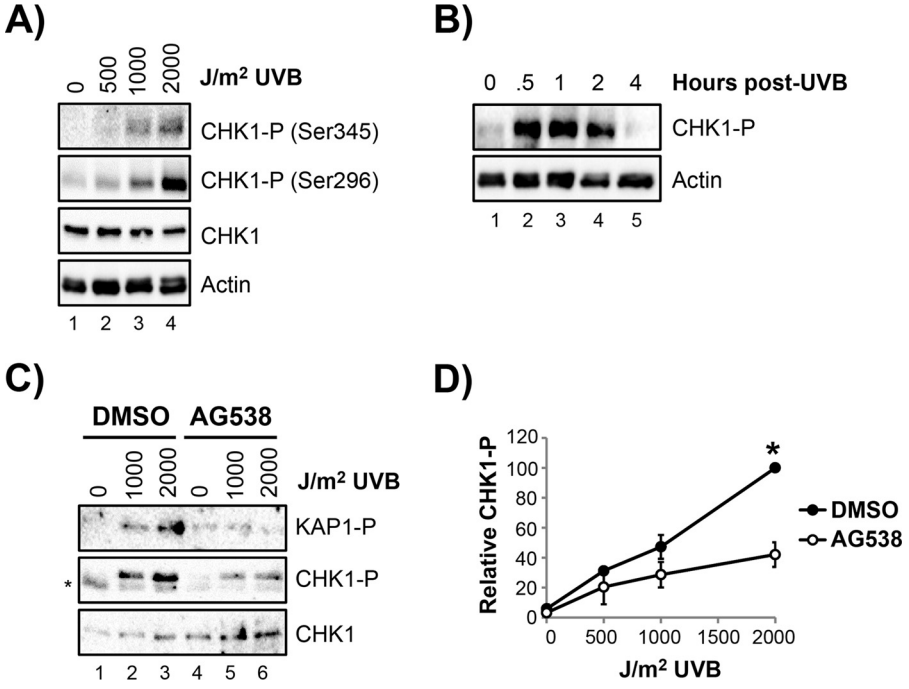

FIGURE 4. **Inhibition of IGF-1R in human skin explants *ex vivo* partially blocks UVB-induced ATR-CHK1 signaling.** *A*, human skin epidermis from routine abdominoplasty surgeries was separated from the dermis by vacuum for 60–90 min prior to exposure to the indicated fluences of UVB light. The epidermis was isolated and then analyzed by immunoblotting. *B*, human epidermis was processed as in *A*, exposed to 1000 J/m^2 of UVB radiation, and harvested at the indicated time points for immunoblotting analysis. *C*, solutions of DMSO or AG538 were applied to human abdominoplasty skin for 0.5 h prior to exposure to the indicated dose of UVB radiation. The epidermis was harvested 0.5–1 h later and analyzed by immunoblotting. *D*, the mean (± S.E.) level of CHK1 phosphorylation from two to four independent experiments at each UVB dose. The *asterisks* indicate that CHK1 phosphorylation in the AG538-treated epidermis is significantly different from in the DMSO-treated epidermis (*, $p < 0.05$).

nents of the nucleotide excision repair system, in which it facilitates damage recognition and coordinates various enzymatic activities (43, 44). Thus, defective recruitment of RPA to UVB-damaged chromatin may also contribute to the previously reported inhibition of UVB photoproduct removal in keratinocytes with an inactive IGF-1R (39, 40).

The mechanism by which RPA is deregulated in cells with an inactive IGF-1R remains to be determined. However, we note that an insufficient supply of RPA has been recognized as a contributor to a variety of negative cellular phenotypes in response to replication-associated genotoxic stress (53, 56–60). Thus, the generation of endogenous replicative

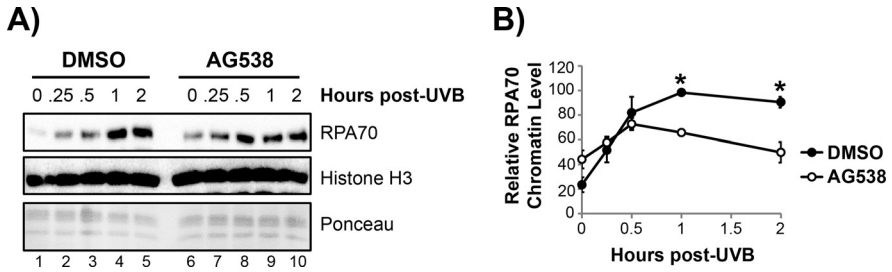

FIGURE 5. **RPA accumulation on chromatin after UVB irradiation is partially abrogated by inhibition of the IGF-1/IGF-1R signaling.** *A*, N-TERTs treated with DMSO or AG538 were exposed to 125 J/m^2 of UVB radiation. At the indicated time points, cells were harvested for fractionation and enrichment of chromatin-associated proteins, which were analyzed by immunoblotting with antibodies against the 70-kDa subunit of RPA (*RPA70*) or histone H3. The blot was also stained with Ponceau S prior to immunoblotting to detect total histones and ensure equal loading. *B*, quantitation of chromatin-bound RPA. Experiments performed as in *A* were quantified so that the maximal RPA70 signal on each experimental blot was set to an arbitrary value of 100, and all other samples were normalized to this value. Data show the mean ± S.E. from four independent experiments. The *asterisks* indicate the time points at which chromatin-bound RPA was significantly reduced in the AG538-treated cells relative to the DMSO-treated cells (*, $p < 0.05$).

stress by aberrant IGF-1 signaling could, in principle, limit the amount of RPA available for various UV DNA damage responses, including nucleotide excision repair and ATR-CHK1 signaling.

Nonetheless, the results presented here demonstrate that IGF-1R status impacts a second DNA damage response (the suppression of DNA synthesis) following UVB exposure. A schematic summarizing these findings is provided in Fig. 6*A*. Together, nucleotide excision repair and ATR-CHK1-mediated inhibition of DNA replication likely cooperate to prevent mutagenesis and promote cell survival in cells with an activate IGF-1R. Under conditions of more extensive DNA damage, ATR may also facilitate a protective senescence (54, 55) in cells with an intact IGF-1/IGF-1R system (42) that contributes to the prevention of carcinogenesis. However, when IGF-1 levels are deficient, the decreased rate of photoproduct removal by nucleotide excision repair coupled with an abrogation of ATR-CHK1 signaling and replication inhibition may increase the risk of mutagenesis, decrease the likelihood of senescence, and, ultimately, promote skin carcinogenesis (Fig. 6*B*).

The fact that IGF-1 expression is low in the skin of geriatric patients (37) has important implications for understanding the mechanisms and origins of non-melanoma skin cancers in geriatric patients. Defects in both DNA repair and DNA damage signaling because of low IGF-1 may therefore contribute to the risk of developing skin cancer. Interestingly, dermal rejuvenation approaches have been shown to increase IGF-1 levels in the skin and to reduce the number of proliferating, basal keratinocytes with unrepaired DNA photoproducts (61–63). Thus, understanding the molecular mechanisms of DNA repair and DNA damage signaling in the context of the IGF-1 pathway and aging may offer new approaches for preventing NMSCs in susceptible individuals.

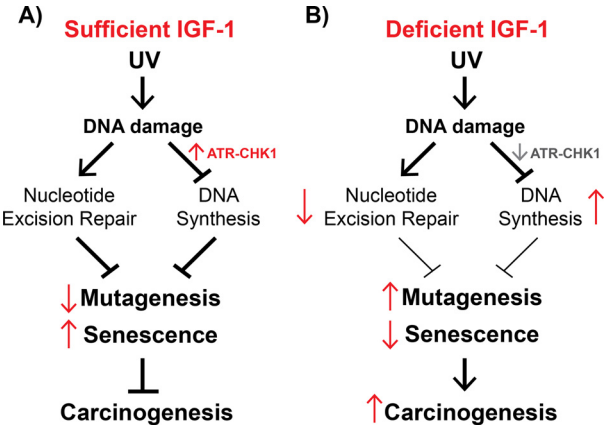

FIGURE 6. **Model describing how deficient IGF-1/IGF-1R signaling impacts the cellular response to UVB-induced DNA damage.** *A*, exposure of cells to UVB wavelengths of light results in the formation of UV photoproducts in DNA. In the presence of sufficient levels of IGF-1, this DNA damage can be repaired by nucleotide excision repair but also activates ATR-CHK1 signaling to suppress DNA synthesis. Together, DNA repair and DNA synthesis inhibition are expected to limit mutagenesis and, subsequently, carcinogenesis. Under conditions of more extensive DNA damage, cells may also undergo a protective senescence to prevent carcinogenesis. *B*, when IGF-1 levels are deficient and IGF-1R signaling is suboptimal, the nucleotide excision repair rate is reduced, and DNA synthesis fails to be properly suppressed. Defects in these processes may therefore increase the risk of mutagenesis, decrease the likelihood of cells undergoing senescence, and, subsequently, promote carcinogenesis.

Experimental Procedures

Cell Culture—Primary keratinocytes from human neonatal foreskins and telomerase-immortalized keratinocytes (N-TERTs) (45) were cultured in EpiLife medium supplemented with human keratinocyte growth supplement (Thermo Fisher Scientific) and penicillin/streptomycin (Gibco). Experiments with cells deprived of IGF-1 were grown for 18–24 h in the same medium but with IGF-1 omitted from the human keratinocyte growth supplement. Recombinant IGF-1 was added to the culture medium at the indicated final concentration 1 h prior to UVB exposure. The IGF-1R inhibitors I-OMe-Tryphostin AG538 (Sigma) and picropodophyllin (PPP, Selleck Chem) were dissolved in DMSO and added to medium at 10 μM or 5 μM, respectively, 1 h prior to UVB irradiation. Cells were exposed to the indicated fluences of UVB radiation as described previously (37) using a Philips F20T12 UV bulb at a dose rate of 5 J/m^2/s.

Human Skin Epidermis—The epidermis of human skin from abdominoplasty procedures was separated from the underlying dermis by inducing suction blisters for 60–90 min with 20-ml syringes placed on the skin and attached via tubing to vacuum pumps. The IGF-1R inhibitor AG538 (20 μM in DMSO) or DMSO (vehicle) was then dispensed topically onto the skin, which, after a 30-min incubation, was subsequently exposed to the indicated fluences of UVB radiation. Following the various treatments, the epidermis was removed from the skin using forceps, washed twice with cold PBS, and then stored at −80 °C.

Epidermal lysates were prepared by sonicating the epidermis in radioimmune precipitation buffer and then processed for immunoblotting as described below.

Cell Lysis and Analysis of Chromatin-associated Proteins—Media from treated/irradiated cells was discarded, and the cells were then washed once with cold PBS before scraping the cells from the plate in cold PBS. The cells were pelleted by gentle centrifugation and either frozen and stored at −80 °C or directly lysed for 20 min on ice in radioimmune precipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS; Teknova) containing a protease inhibitor mixture (Sigma), 1 mM DTT, 0.1 mM PMSF, 10 mM NaF, 1 mM Na_2VO_3, and 10 mM glycerophosphate. Samples were briefly subjected to five to six pulses with an F60 Sonic Dismembrator (Fisher Scientific) before centrifugation in a microcentrifuge for 15 min at maximum speed. The soluble cell lysates were then transferred to new tubes. For the analysis of chromatin-associated proteins, cells were extracted three to four times with buffer A (10 mM HEPES-KOH (pH 7.6), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, and 0.1% Triton X-100 containing 1 mM DTT and protease and phosphatase inhibitors) to fully separate soluble proteins from chromatin-associated proteins, as described previously (46). The Triton-resistant chromatin fraction was subsequently resuspended in buffer A, supplemented with 1× SDS-PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 5% glycerol, 100 mM DTT, 1% SDS, and 0.005% bromphenol blue), and sonicated to shear the genomic DNA.

Immunoblotting—Equivalent amounts of soluble or insoluble cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and then probed by immunoblotting using standard procedures. Primary antibodies included antibodies against CHK1 (sc-8408) and actin (I-19) from Santa Cruz Biotechnology; RPA70 (A300-421A) and phospho-KAP-1 (Ser-824; A300-767A) from Bethyl Laboratories; and phospho-CHK1 (Ser-345; 2348), phospho-CHK1 (Ser-296; 2349), and histone H3 (3638) from Cell Signaling Technology. All primary antibodies were used at a 1:1000 or 1:2000 dilution in 1× TBST (50 mM Tris-HCl (pH 7.4), 135 mM NaCl, and 0.1% Tween 20). Secondary antibodies included horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG from GE Amersham Biosciences, Pierce, or Invitrogen and anti-goat IgG (sc-2020) from Sigma. Chemiluminescence was visualized with Clarity Western ECL substrate (Bio-Rad) or SuperSignal West Femto substrate (Thermo Scientific) using a Molecular Imager Chemi-Doc XRS+ system (Bio-Rad). Chemiluminescent signals within the linear range of detection were quantified using ImageQuant software (GE Healthcare). For each soluble lysate immunoblot, the phospho-CHK1 signal was quantified and normalized to total CHK1 levels in the same protein lysate, and the maximum signal for each blot was set to an arbitrary value of 100. All other phospho-CHK1/CHK1 ratios were then normalized to this value for each immunoblot. All experiments analyzing ATR-CHK1 signaling were repeated three to four times, and the mean ± S.E. of the phospho-CHK1protein/CHK1 was determined and plotted. Statistically significant differences were determined using a Student's *t* test.

Immuno-Dot Blot Analysis—DNA synthesis was measured with an immuno-dot blot assay (47). BrdU was added to the cell culture medium at a final concentration of 10 μg/ml 30 min prior to cell harvesting at the indicated time points following UVB irradiation. Cells were then washed in cold PBS, scraped from the plate, centrifuged at 1600 × *g* in a microcentrifuge for 5 min at 4 °C, and then stored at −80 °C until ready for further processing. Genomic DNA was next purified with either a QIAamp DNA Mini Kit (Qiagen) or a GenElute Mammalian Genomic DNA Miniprep kit (Sigma) and quantified using a Nanodrop spectrophotometer and/or agarose gel electrophoresis. Genomic DNA (100–200 ng) diluted in water was heated at 90 °C for 10 min before neutralization with an equal volume of 2 M ammonium acetate and incubation on ice for 10 min. DNA was immobilized on a nitrocellulose membrane with a Hybri-Dot manifold (Bethesda Research Laboratories) and baked at 80 °C for 90 min. Blots were blocked in 5% milk in TBST and probed overnight with an anti-BrdU antibody (Sigma, B2531). Blots were reprobed with anti-ssDNA antibody (Millipore, MAB3034) to ensure equal loading of DNA. Chemiluminescent detection was performed as described above, and all signals were normalized to non-irradiated control samples. All experiments were repeated between two and ten times (as indicated), and representative results are presented. A Student's *t* test was used to determine statistically significant differences between treatment groups.

Author Contributions—M. G. K. conceived the idea for this project and carried out the experiments with input from D. F. S. and J. B. T. R. S. provided abdominoplasty skin. M. G. K., D. F. S., R. S., and J. B. T. analyzed the results, and M. G. K. wrote the paper.

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